RT-qPCR using the PCR Optimization Kit

Step-by-step instructions for RT-qPCR using preformulated buffers.

Katharine D. Hoffmann and Leta S. Steffen
Promega Corporation

Materials Required

- PCR Optimization Kit (Cat.# D2381): The PCR Optimization Kit contains PCR Buffers A-H, $MgCl_2$, Nuclease-Free Water, and GoTaq® MDx Hot Start Polymerase
- GoScript® Reverse Transcriptase (Cat.# A5003)
- Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511)
- Fluorescent dsDNA-binding dye (e.g., BRYT™ Green or SYBR Green I) and CXR Reference Dye (for real-time instrument runs that require a passive reference dye)
- Target-specific primers
- QPCR Human Reference Total RNA (Agilent Technologies #750500)

With the addition of reverse transcriptase and RNase inhibitor, the PCR Optimization Kit reagents support RT-PCR and RT-qPCR.

Successful RT-qPCR is dependent on an optimal combination of chemistry, primer design, template quality, and cycling parameters. The PCR Optimization Kit provides a range of buffer formulations that make it easy to determine optimal amplification conditions. With the addition of GoScript® Reverse Transcriptase and RNasin® Ribonuclease Inhibitor, these buffers can also be used to optimize RT-qPCR conditions. This article demonstrates RT-qPCR using the buffers supplied in the PCR Optimization Kit and provides example functional data.

Introduction

Reverse transcription qPCR (RT-qPCR) is a real-time PCR method that enables sensitive amplification and quantification of an RNA target. RT-qPCR may be used for assessing gene expression or viral load, among other applications.

In 1-Step RT-qPCR, reverse transcription of an RNA target is followed by DNA polymerase-mediated amplification in a single tube. Both processes share a single set of gene-specific primers. Master mixes designed to support 1-Step RT-qPCR must be formulated to enable activity of both the reverse transcriptase and the DNA polymerase, be capable of inhibiting RNA degradation, and support fluorescent detection chemistry.

This article describes how to use the Promega PCR Optimization Kit for 1-Step RT-qPCR. The PCR Optimization Kit provides a series of pre-formulated buffers designed to streamline assay development and optimize PCR-based applications. With the addition of GoScript™ Reverse Transcriptase, Recombinant RNasin® Ribonuclease Inhibitor and dsDNA binding dye, the optimization kit buffers are also suitable for 1-Step RT-qPCR applications.

Methods

Reaction Set Up: PCR Buffers A-H were tested for amplification of a 139bp β2 microglobulin target (B2M, NM_004048.2) from serially diluted human total RNA using dye-based 1-Step RT-qPCR. Each 5X PCR Buffer (containing dNTPs) was used to prepare an intermediate 2X master mix (on ice) containing polymerase, reverse transcriptase, $MgCl_2$, and fluorescent

dyes, to model a custom master mix. After combination of equal volumes of RNA and Master Mix, the final 1X reaction components included 1X PCR Buffer, 0.05U/µl GoTaq® Hot Start Polymerase, 0.2U/µl GoScript® Reverse Transcriptase, 1U/µl Recombinant RNasin® Ribonuclease Inhibitor, 2mM $MgCl_2$, 1X BRYT® Green Dye, 30nM CXR (passive reference dye) and 200nM forward and reverse primers in a 25µl reaction.

Primers: B2M primer sequences (F) AAAGCAAGCAAGCAGAATTTGG, and (R) GTATGCCTGCCGTGTGAAC (BioSearch Technologies), were shown to be specific to a 139bp target in the human RefSeq mRNA database using Primer-Blast1. The only match within the genome reference assembly is >2000bp and is unlikely to amplify.

RNA Standards: Human Total RNA (Agilent QPCR Human Reference Total RNA) was serially diluted 5-fold from 25ng–1.6pg per reaction in Nuclease-Free Water.

Cycling Parameters: All samples and no-template controls were amplified in quadruplicate reactions on an Applied Biosystems 7500 Fast Real-Time PCR System in fast mode with the following cycling conditions: 15 minutes at 37°C; 10 minutes with minutes at 95°C; 40 cycles of 10 seconds at 95°C, 1 minute at 62°C; default dissociation (melt curve). Optimum reaction conditions and cycling parameters depend on the combination of chemistry, primers, and target. Optimum conditions for other assays may differ from the conditions used in this report.

Data Analysis: Data were analyzed using 7500 Software v 2.0.6 (Applied Biosystems) with automatic baseline and threshold methods except as indicated. Baselines were verified using the linear amplification view, while thresholds were verified using the semi-log amplification view. Assay linearity and efficiency were assessed for the standard curve of RNA serial dilutions.

Optimum reaction conditions and cycling parameters depend on the combination of chemistry, primers and target.

Results

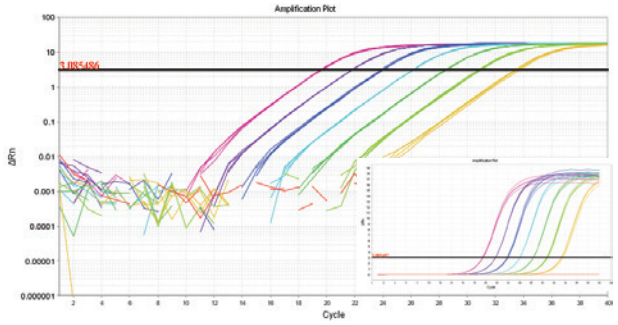
Promega PCR Optimization Kit Buffers A-H were tested for their ability to support 1-Step RT-qPCR under typical user conditions. All eight PCR Buffers supported amplification at all RNA concentrations tested. Amplification in no-template control reactions was seen only with PCR Buffer H, and likely represents primer artifacts (e.g., primer-dimer) as indicated by the low product melting temperature (Figure.1).

Automatic baseline and threshold values were determined by the 7500 Software for each buffer tested with the following exceptions: For PCR Buffer G, the software-determined baseline settings for one NTC reaction were manually increased from cycles 1–3 to cycles 3–15, consistent with typical baseline settings. For PCR Buffer H, the software-determined threshold setting was used but is at the very low end of the exponential phase, apparently caused by the non-specific amplification in NTC wells.

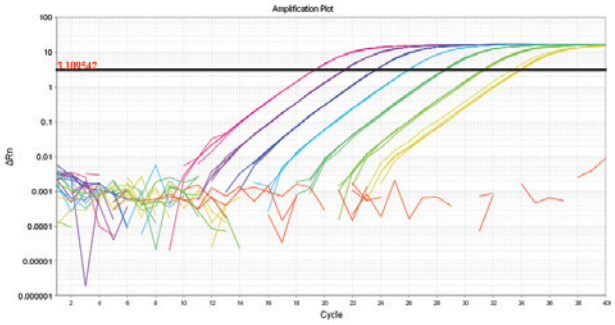
Threshold values are expected to change with dye, chemistry and assays used, so each experiment must be analyzed separately for appropriate quantitation. Assays were linear between 1.6pg and 25ng of RNA, as indicated by R^2 values ≥0.980 in all eight PCR Buffers. Assay efficiency was within the recommended range of 90–110% in seven of the eight buffers (2).

Melt analysis demonstrated a single melt peak in each buffer, consistent with specific amplification of a single product (aside from primer artifacts in PCR Buffer H NTC reactions). Within any given buffer, melting temperatures vary by ≤0.16% CV. As expected, the melting temperature of the same amplicon varies between buffers, due to the influence of PCR buffer salts and additives on DNA duplex stability. Melting temperatures for each buffer are given in Table 1. These range from as low as 77.17°C in PCR Buffer F to 82.75°C in PCR Buffer D. Generally, product melting temperature shifts are expected to mimic oligo annealing temperature shifts. The low melting temperature of the product in PCR Buffer F may indicate reduced DNA duplex stability. Optimization of the assay in this particular buffer would therefore start with testing of reduced oligo annealing temperatures in qPCR cycling.

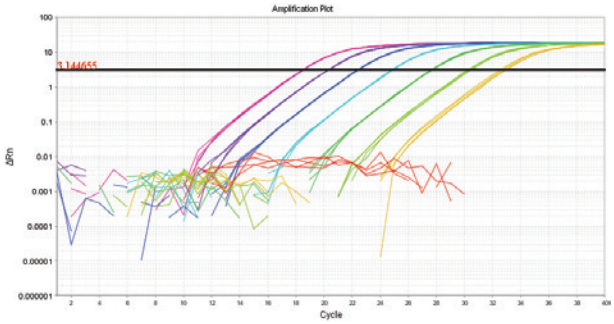
PCR Buffer A



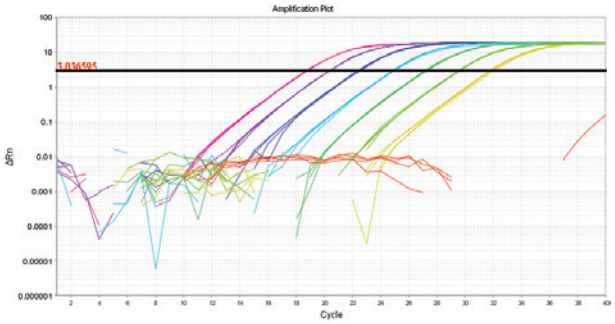
PCR Buffer B



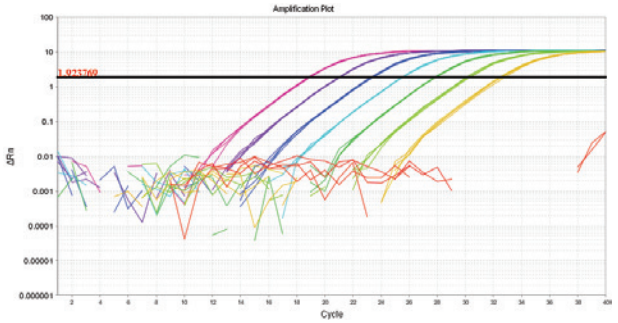
PCR Buffer C



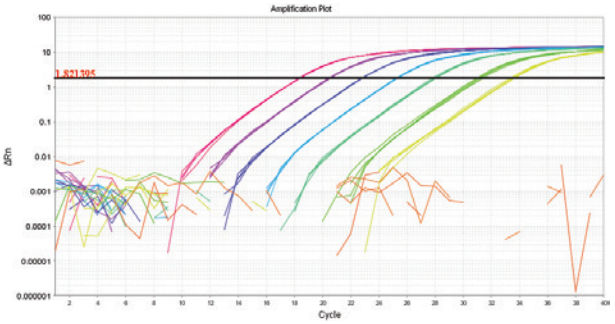
PCR Buffer D



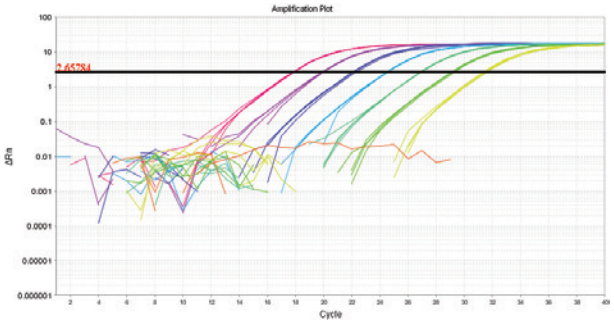
PCR Buffer E



PCR Buffer F



PCR Buffer G



PCR Buffer H

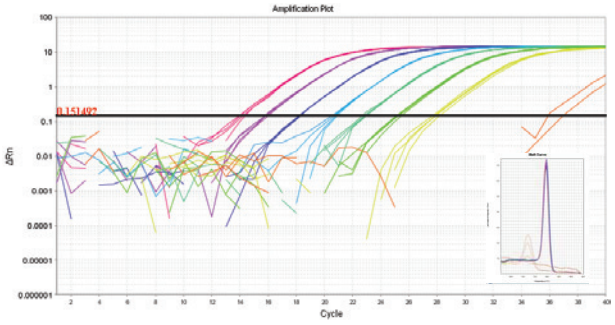


Figure 1. Semi-log amplification plots for 1-Step RT-qPCR amplification of the B2M target from serially diluted human RNA in PCR Buffers A-H. Software-determined thresholds are indicated with the horizontal black line. An example linear amplification plot is shown as an inset for PCR Buffer A. NTC reactions (2 of 4 reactions) in PCR Buffer H show non-specific amplification as indicated by the low melting temperature (red traces, inset melt curve).

Table 1. Summary statistics for 1-Step RT-qPCR of a 139bp B2M amplicon using PCR Buffers A-H.

	Specificity		Linearity	Sensitivity		Efficiency	
Buffer	NTC	Product Tm, °C (%CV)	R^2	Cq (Std) of 1.6pg	Y-Intercept	(Slope)	Threshold
PCR Buffer A	0/4	80.97 (0.16)	0.998	33.45 (0.15)	33.84	102% (-3.29)	3.09
PCR Buffer B	0/4	80.13 (0.16)	0.998	33.69 (0.32)	34.20	94% (-3.47)	3.11
PCR Buffer C	0/4	82.46 (0.16)	0.995	32.75 (0.21)	33.26	94% (-3.49)	3.14
PCR Buffer D	0/4	82.75 (0.15)	0.997	31.90 (0.12)	32.37	106% (-3.20)	3.04
PCR Buffer E	0/4	80.58 (0.16)	0.999	32.56 (0.16)	33.12	102% (-3.27)	1.92
PCR Buffer F	0/4	77.17 (0.11)	0.997	33.45 (0.15)	34.08	87% (-3.67)	1.82
PCR Buffer G	0/4	80.11 (0.12)	0.999	31.41 (0.10)	32.04	102% (-3.27)	2.66
PCR Buffer H	2/4*	79.49 (0.12)	0.997	28.00 (0.24)	28.43	101% (-3.30)	0.15

Std = standard deviation; NTC = number of no template control reactions with amplification; Tm = melting temperature; %CV = coefficient of variation.
*Non-specific amplification, indicated by broad, low-Tm peak in the melting profile. Cq values are >8 cycles later than 1.6pg standards.

The melting temperature of a single product is expected to vary between buffers, due to the influence of PCR buffer salts and additives on DNA duplex stability.

Conclusions

The PCR Optimization Kit offers a breadth of buffer formulations appropriate for diverse endpoint and real-time PCR applications that can be used for manufacture of custom master mixes. With the addition of reverse transcriptase, RNase inhibitor and fluorescent dyes, these reagents also support RT-qPCR applications.

References

1. Ye, J. *et al.* (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134, doi:10.1186/1471-2105-13-134.
2. Bustin, S.A. *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611-622, doi:10.1373/clinchem.2008.112797.

Ordering Information

Product	Cat.#
PCR Optimization Kit	D2381
GoScript™ Reverse Transcriptase	A5003
Recombinant RNasin® Ribonuclease Inhibitor	N2511

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